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TITLE: Role of the Adherens Junction Protein Fascin in the Regulation of Tight Junction Permeability in the Mouse Mammary Gland

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An adenovirus-based gene delivery to the mouse mammary gland and cultured cells was developed. This system was used to express a truncation mutant of the tight junction protein occludin. Occludin and the tight junction complex proteins ZO-1 and claudin-1 left the tight junction following transgene expression, demonstrating tight junction disruption. Truncated occludin expression caused apoptosis in the mouse mammary gland epithelium and in cultured cells. The apoptotic mediator caspase-8 was activated within 24 hours of viral transduction. The caspase-8 downstream target caspase-3 was proteolytically activated and beta-catenin, one of many caspase-3 downstream targets, was cleaved within 24 hours of viral transduction. Caspase-8 is the chief regulatory caspase of the death inducing signaling complex (DISC) pathway. Early activation of caspase-8 suggests DISC mediation of the observed apoptosis. PTEN is a regulatory lipid phosphatase whose activity has been shown to attenuate Akt activity, correlating with DISC activation. PTEN localizes to the tight junction of epithelial cell monolayers. This has led to our current hypothesis: "PTEN is released from the tight junction and re-distributes to lipid rafts containing DISC precursors. PTEN attenuates Akt activity in these rafts allowing DISC activation."

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Introduction: The tight junction is an array of anastomosing proteinaceous fibers lying in the apicolateral membrane region of adjacent epithelial cells. These fibers lie in parallel along their lengths and are thought to interact between cells via hetero and homophilic interactions among the occludin and claudin proteins composing them. The intracellular C-termini of occludin and the claudins have been shown to interact with membrane associated guanylate kinases (MAGUKs), such as ZO-1. Tight junction associated MAGUKs and other proteins form an intracellular plaque that runs continuously along the tight junction fibrils. This plaque connects transmembrane proteins in tight junction fibrils to actin stress fibers, which in turn connect to the perijunctional actin ring. The MAGUK family members also serve to localize regulatory molecules to the tight junction, largely through interactions mediated by the PDZ domains of MAGUK proteins (see Figure 1 page 7).

The cadherin based adherens junction occurs basal to the tight junction and, like the tight junction, runs continuously along the apicolateral border of adjacent epithelial cells. An intracellular plaque runs along the intracellular C-termini of the cadherins. This plaque is comprised of catenin protein family members. The catenin-based plaque connects the transmembrane cadherins to actin stress fibers that in turn connect to the perijunctional actin ring.

The tight junction, the adherens junction and the perijunctional actin ring comprise the apical junctional complex. The architecture of the apical junction complex is perturbed in breast cancer. Claudins, ZO-1, the tight junction associated regulatory protein PTEN, cadherins, and the catenins are irregularly expressed in breast cancer. The perijunctional actin ring is poorly defined in breast cancer.

A great deal of scientific research has been devoted to intracellular signaling downstream of cadherin binding. It has been shown that proper cadherin mediated cell/cell adhesion is necessary for the survival of normal epithelial cells. Based upon preliminary results from this project, I hypothesize that there is also intracellular signaling from the tight junction and that proper occludin/claudin mediated cell/cell adhesion is necessary for normal mammary epithelial cell survival.

The cells comprising a metastatic breast tumor are by definition able to survive loss of proper cell/cell adhesion. Understanding the survival dependency of healthy epithelial cells upon proper cell/cell adhesion will be of great value in fighting breast cancer. With a better understanding of the mechanisms that cause normal cells to die when unable to maintain proper cell/cell contact, it may be possible to develop therapies that only target cancerous cells already showing irregular cell/cell binding.

This project is currently testing the hypothesis that normal mammary epithelial cells require occludin mediated tight junction cell/cell adhesion for survival. The role of the death inducing signaling complex (DISC) in mediating apoptosis downstream of tight junction disruption is being studied.

During the previous grant cycle an adenovirus gene delivery system was developed and used to deliver truncated occludin to cells in culture and to the mouse mammary gland epithelium. This "DeltaOcc" occludin construct is comprised of the C-terminal half of the occludin molecule, with the last half of the second extracellular loop fused to the FLAG epitope tag at the new N-terminus. This virus also encodes the green fluorescent protein as a separate reporter gene. Cells expressing the DeltaOcc transgene underwent disruption of the tight junction followed by apoptosis (see 2003 report).

Since that time, I have been studying the behavior of known apoptotic intermediates in apoptosis downstream of tight junction disruption. I have proposed a new model now being tested whereby the lipid phosphatase PTEN is released from sequestration at the tight junction and plays a positive role in DISC mediated apoptosis.

Body:

1. Truncated occludin expression causes tight junction disruption: CIT3 mouse mammary epithelial cells express an N-terminally truncated, N-terminally FLAG epitope tagged, occludin transgene when transduced with the "DeltaOcc" adenovirus encoding this construct. Figure 2 (page 8) shows that the DeltaOcc transgene localized at the tight junction with the tight junction protein ZO-1 at 18 hours post transduction. Figure 3 (page

9) shows that the DeltaOcc transgene localized at the tight junction with ZO-1 and also showed intracellular, vessicular distribution at 48 hours post viral transduction. ZO-1 distributed to the tight junction and to the intracellular DeltaOcc positive vesicles at 48 hours post transduction. A similar staining was performed using an anti-FLAG antibody and an antibody against the tight junction protein claudin-1. This staining also indicated an initial localization of DeltaOcc to the tight junction, followed by tight junction disruption (not shown).

Conclusion: I have developed an adenovirus expressing an occludin truncation mutant that results in tight junction disruption in transduced cells.

2. Tight junction disruption correlates with increased apoptosis: It became apparent that cells transduced by DeltaOcc underwent apoptosis. In order to verify apoptosis, CIT3 mammary epithelial cells were transduced with the DeltaOcc virus and processed for TUNEL staining. TUNEL staining fluorescently labels the disrupted, condensed chromosomal DNA found during apoptosis. Figure 4 (page 10) shows the presence of many TUNEL positive nuclei in DeltaOcc transduced cells, indicating apoptosis. Control cells transduced with an adenovirus encoding only the GFP reporter gene are free of TUNEL positive nuclei, indicating that they are not apoptotic.

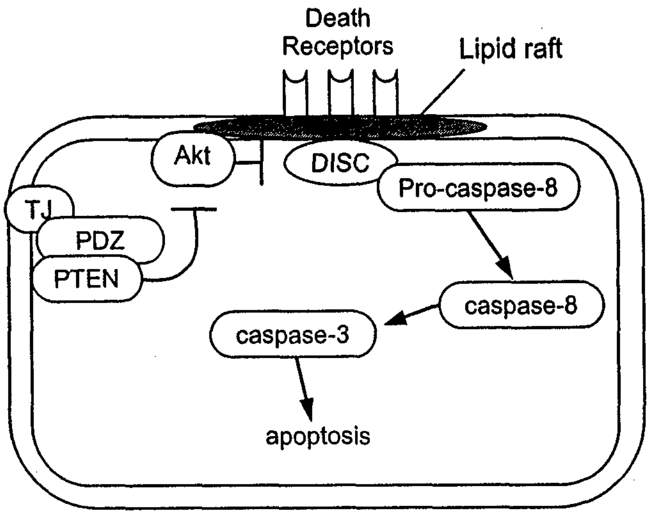
In order to verify that tight junction disruption causes apoptosis in the mouse mammary gland, control adenovirus encoding only GFP or DeltaOcc adenovirus was injected through the ductal opening into the mammary lumen of mice during late pregnancy. Figure 5 (page 11) shows that the mouse mammary epithelium tolerates transduction with GFP control adenovirus. Panel "A" shows a frozen section through two highly transduced alveoli. These alveoli are morphologically normal and cytoplasmic lipid droplets are present within transduced cells. Cytoplasmic lipid droplets are a normal and necessary part of milk fat secretion. Further staining also showed normal distribution of casein, the most abundant milk protein, in the secretory apparatus of cells transduced with GFP only adenovirus (not shown). In contrast, the mouse mammary gland does not tolerate the presence of cells transduced by the DeltaOcc adenovirus. Panel "B" in figure 5 shows that DeltaOcc positive cells have irregular morphology and are shed into the mammary lumen. These cells appear to be undergoing blebbing and nuclear condensation, integral parts of anoikis or epithelial cell apoptosis.

In order to further elucidate the apoptotic response in DeltaOcc treated cells, I performed western blotting on apoptotic intermediates. Figure 6 (page 12) shows the presence of the active, proteolytic fragments of both caspase-8 (18kd) and caspase-3 (19kd) following transduction with DeltaOcc adenovirus. Caspase-8 and caspase-3 are not proteolytically activated in control cells transduced with the GFP control adenovirus or in non-transduced cells grown in parallel. All lysates show the presence of the predicted higher molecular weight precursors. Other western blots showed that the caspase-3 proteolytic target protein beta-catenin is cleaved in lysates from DeltaOcc transduced cells and is not cleaved in control cells (not shown).

Conclusion: I have demonstrated that apoptosis occurs in cells expressing the DeltaOcc transgene. This apoptosis is characterized by early activation of the regulatory caspase-8. Early activation of caspase-8 indicates that the observed apoptosis may be mediated by the death inducing signaling complex (DISC).

3. The regulatory lipid phosphatase PTEN localizes to the tight junction in CIT3 cells. PTEN is a lipid phosphatase that localizes to the tight junction via PDZ domain interactions with MAGUK proteins in epithelial cells. Overexpression of PTEN has been shown to trigger DISC mediated apoptosis in the mouse mammary gland. DISC activation occurs in distinct lipid raft domains and results in the proteolytic activation of caspase-8. PTEN has been shown to localize to lipid raft domains where it attenuates Akt activity within these domains. Akt has been shown to prevent DISC activation. These reports have led me to propose the model diagrammed in figure 7 below: I propose that PTEN is released from tight junction sequestration following tight junction disruption and becomes increasingly localized to lipid raft domains with inactive DISC components. PTEN attenuates Akt activity within these rafts, alleviating the Akt repression of DISC activation. DISC is activated

Figure 7: PTEN release is predicted to cause DISC activation.



causing caspase-8 activation. Caspase-8 activates caspase-3, leading to apoptosis. Figure 8 (page 13) shows that PTEN colocalizes at tight junctions with ZO-1 in CIT3 cell lines.

Conclusion: PTEN localizes to tight junctions in mammary epithelial cells and a testable hypothesis has been developed directly linking tight junction disruption with DISC mediated apoptosis.

List of Key Research Accomplishments:

- I have demonstrated my ability to construct adenovirus vectors and use them in the transduction of the mouse mammary gland epithelium pertaining to task 2. Because of the finding that tight junction perturbation causes cell death, task 3 is no longer an advisable line of research.
- I have demonstrated dynamic redistribution of five apical junction proteins (ZO-1, fascin, AF-6, P120-catenin, and β-catenin) in response to perturbation of the tight junction pertaining to task 4 (see 2003 report).
- I have shown that tight junction disruption causes apoptosis characterized by the early activation of caspase-8. This is indicative of DISC mediated apoptosis.
- I have shown that PTEN localizes to the tight junction in mammary epithelial cells. I have devised a testable hypothesis whereby PTEN released from the tight junction initiates DISC mediated apoptosis.
- I have began the study of an occludin knockout transgenic mouse line. These mice do not lactate and I am currently characterizing this defect.
- **Adherex Technologies Inc.** is a biopharmaceutical company that has taken an interest in my research. Adherex is supplying me with peptide agonists against occludin. These peptides have been shown to cause tight junction disruption. I will treat cultured cells and the mouse mammary gland with these peptides and predict apoptosis will occur in agreement with the model diagrammed in figure 7.

List of Reportable Outcomes:

- Poster presentation at DOD Era of Hope 2000 in Atlanta
- Poster presentation at Gordon Research Conference for Mammary Gland Biology 2001 in Bristol N.H.
- December 2001 poster presentation at the American Society for Cell Biology in Washington D.C. (abstract enclosed).
- Russell, T. D., A. Fischer, et al. (2003). "Transduction of the mammary epithelium with adenovirus vectors in vivo." J Virol 77(10): 5801-9.1

Conclusions: An adenovirus based gene delivery system was developed which allows the delivery of target genes to the mouse mammary gland epithelium and to cultured mouse mammary epithelial cells. Transduction of the mammary gland does not induce inflammation or effect tight junction permeability. Transduced cells are morphologically normal and produce milk. This gene delivery system was used to express an N-terminally truncated mutant of the tight junction protein occludin in the mammary gland and in cultured cells. Transgene expression caused tight junction disruption. Transduced cells underwent apoptosis in the mammary gland and in cultured cells.

Figure1: Tight Junction architecture and components

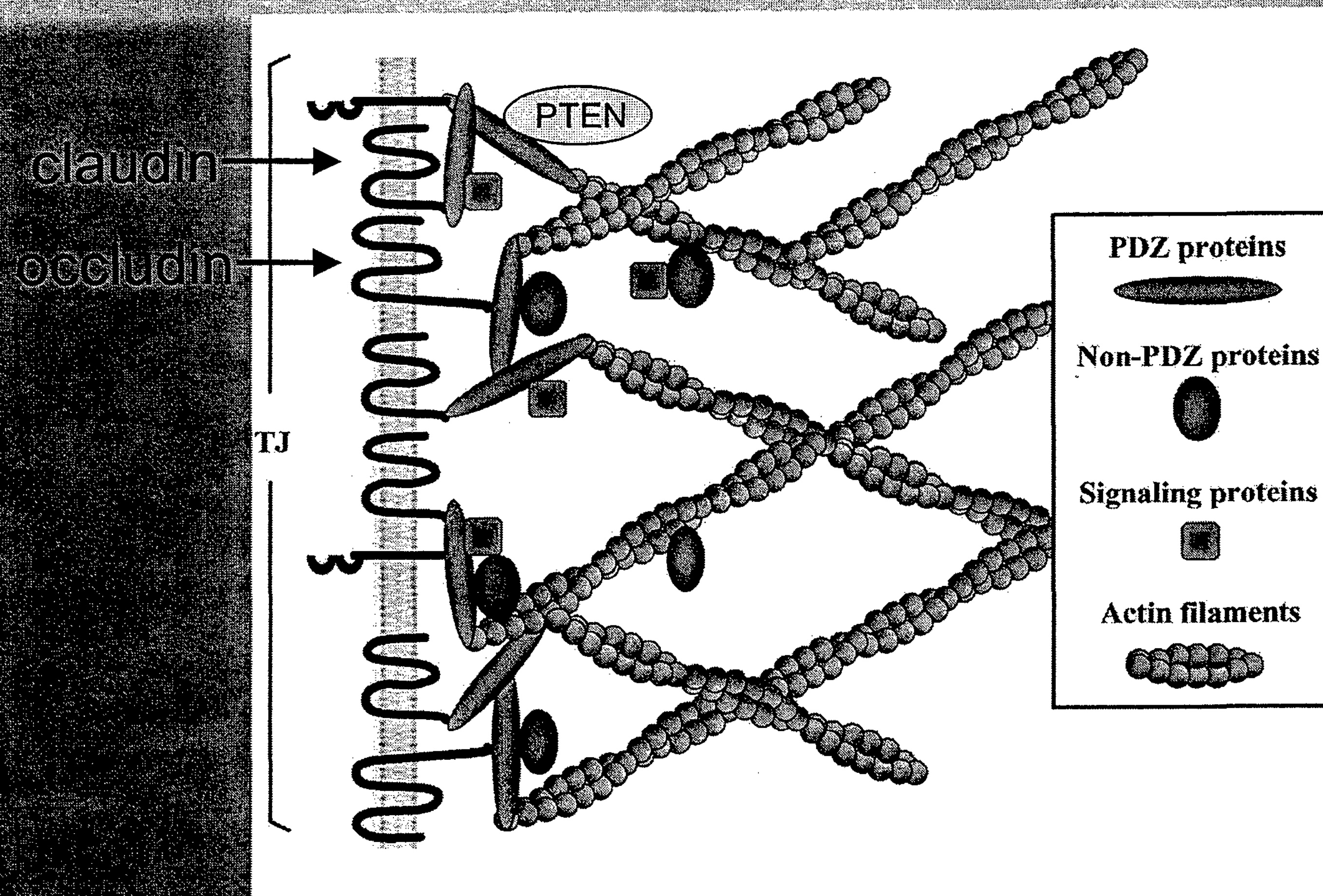
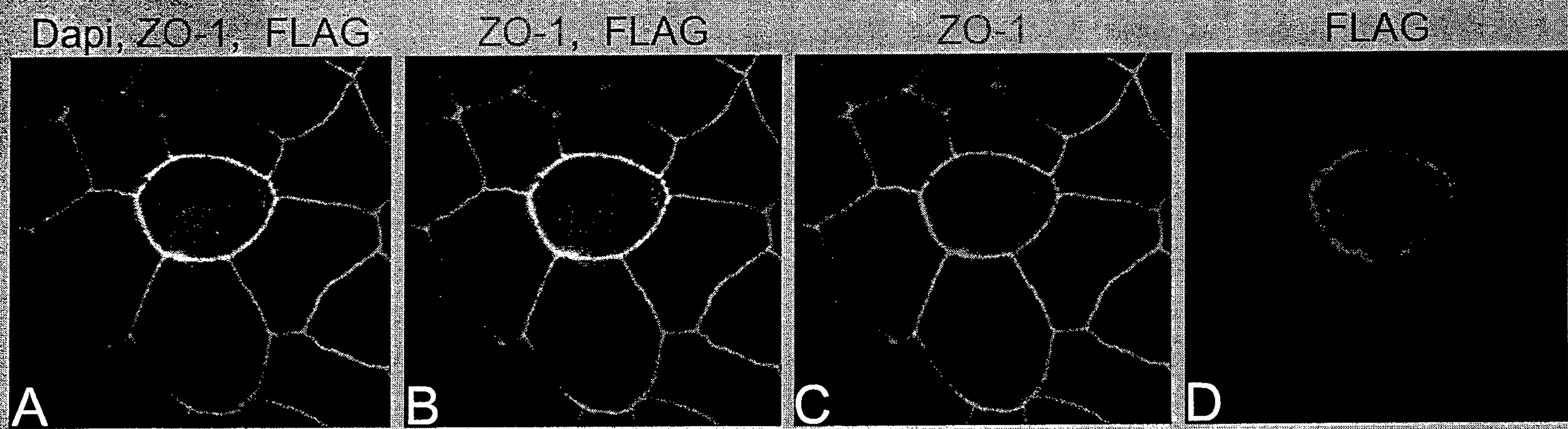


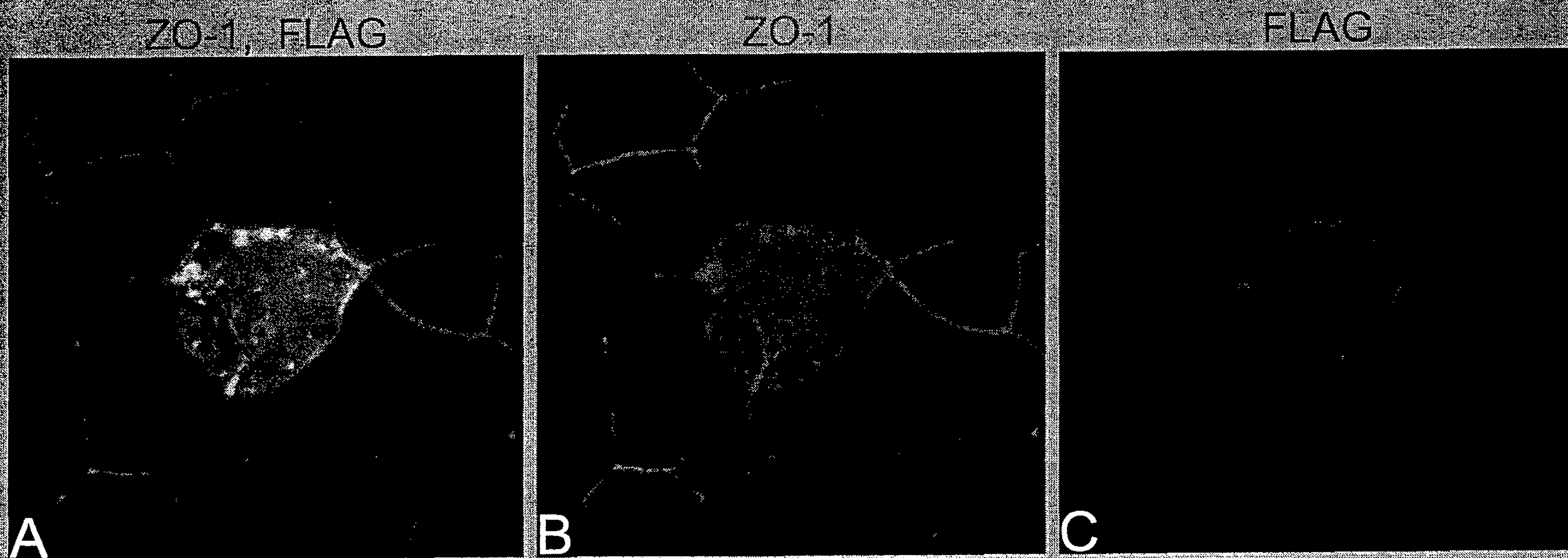
Figure 2. The "DeltaOcc" truncated occludin construct localized at the tight junction 18 hours post transduction.



CIT3 mammary epithelial cells were grown to confluence and transduced with the "DeltaOcc" adenovirus that expresses N-terminally FLAG tagged, truncated occludin. The same virally transduced cell is shown in panels A-D. Staining is as follows: Panel A shows dapi nuclear staining in blue, antibody staining against the tight junction protein ZO-1 in green, and antibody staining against the FLAG epitope tag of the DeltaOcc transgene in red. Panel B shows antibody staining against the tight junction protein ZO-1 in green and antibody staining against the FLAG epitope tag of the DeltaOcc transgene in red. Panel C shows antibody staining against the tight junction protein ZO-1 in green. Panel D shows antibody staining against the FLAG epitope tag of the DeltaOcc transgene in red.

The DeltaOcc transgene localized at the tight junction with ZO-1 at 18 hours post viral transduction.

Figure 3: Delta-occludin expression correlates with tight junction disruption 48 hours post transduction.



CIT3 mammary epithelial cells were grown to confluence and transduced with the "DeltaOcc" adenovirus that expresses N-terminally FLAG tagged, truncated occludin. The same virally transduced cell is shown in panels A-C. Staining is as follows: Panel A shows antibody staining against the tight junction protein ZO-1 in green and antibody staining against the FLAG epitope tag of the DeltaOcc transgene in red. Panel B shows antibody staining against the tight junction protein ZO-1 in green. Panel C shows antibody staining against the FLAG epitope tag of the DeltaOcc transgene in red.

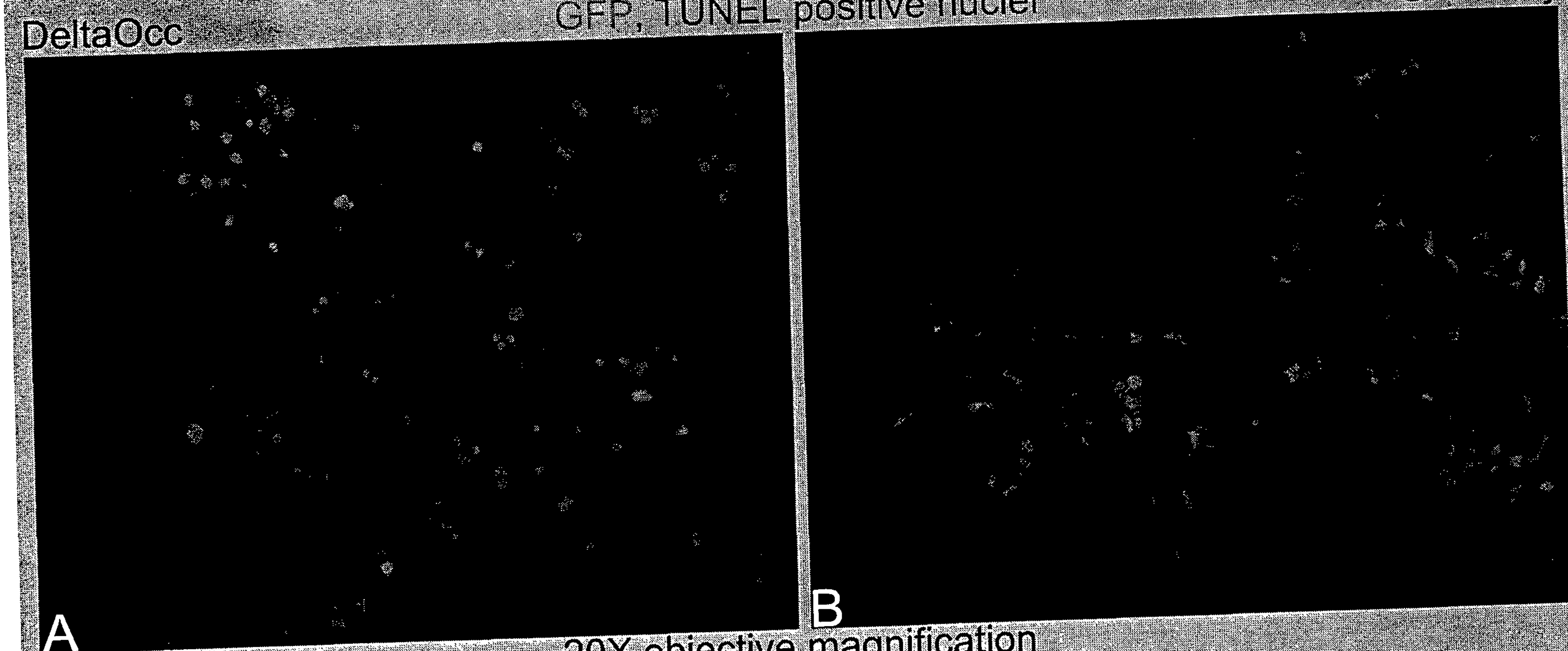
The DeltaOcc transgene localized at the tight junction with ZO-1 and also showed intracellular vessicular distribution at 48 hours post viral transduction. ZO-1 localized at the tight junction and with intracellular DeltaOcc positive vessicles 48 hours post transduction.

Figure 4: TUNEL staining of DeltaOcc expressing CIT3 monolayers indicated apoptosis in cells expressing DeltaOcc.

GFP, TUNEL positive nuclei

DeltaOcc

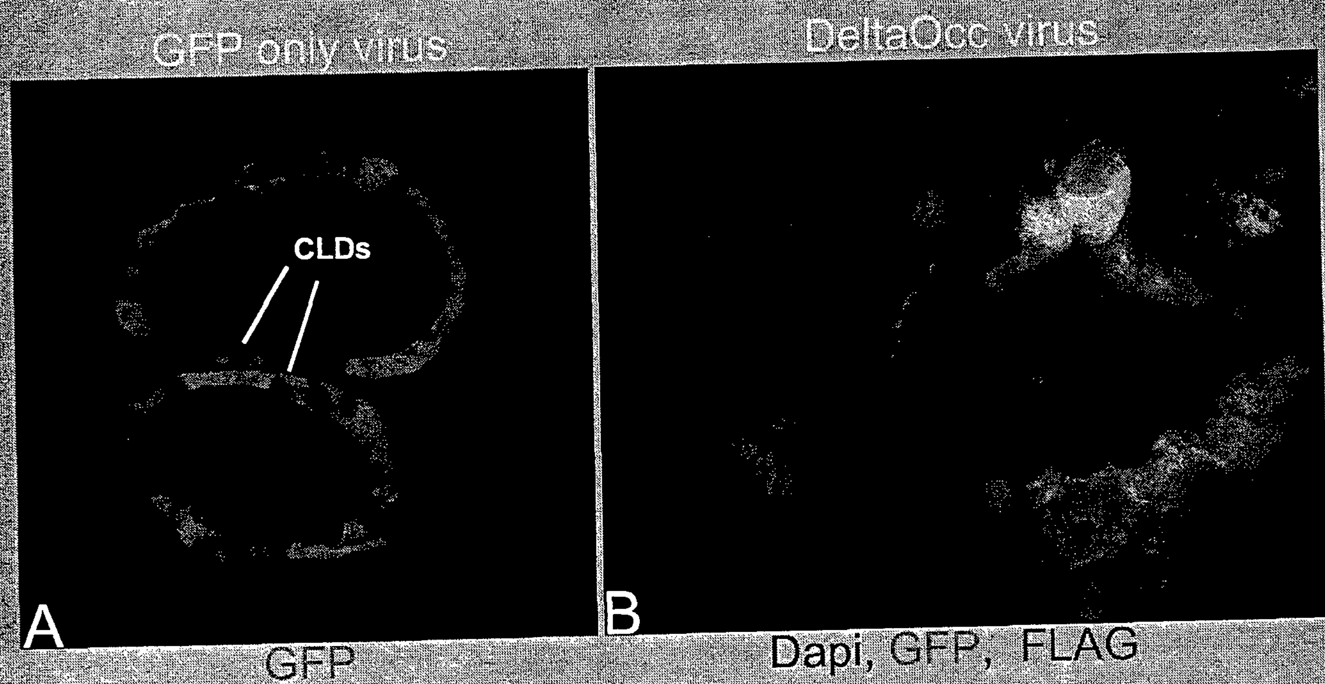
GFP only



20X objective magnification

CIT3 mammary epithelial cells were grown to confluence and transduced with the "DeltaOcc" adenovirus that expresses N-terminally FLAG tagged, truncated occludin and expresses the green fluorescent protein (GFP) as a separate reporter gene (panel A). Control cells were grown in parallel and transduced equally with an adenovirus expressing only the GFP reporter gene (panel B). Cells were processed for TUNEL staining 48 hours following transduction. GFP fluorescence is shown in green. TUNEL staining is shown in red.

Figure 5: DeltaOcc tranduced cells underwent blebbing and were shed into the lumen of the mouse mammary gland.



Adenovirus encoding only GFP (panel A) or the DeltaOcc adenovirus encoding both the DeltaOcc transgene and GFP as a separate protein (panel B) was injected through the ductal opening into the mammary lumen of mice during late pregnancy. Control and experimental mammary glands were then harvested 18 hours following injection and frozen sections were processed for fluorescent microscopy. Panel A shows the GFP fluorescent signal in green. CLDs = Cytoplasmic lipid droplets. Panel B shows the GFP fluorescent signal in green and antibody staining of the FLAG epitope tag in red. Dapi stained nuclei in panel B are shown in blue for morphological reference.

Figure 6: Truncated occludin expression resulted in caspase cleavage.

CIT3 cells were plated at confluent density and virally transduced 18 hours after plating. Cell lysates were collected 24 hours following viral treatment. Lysates were blotted with an antibody against the C-terminus of caspase-8 (top panel) or with an antibody against the C-terminus of caspase-3 (bottom panel). This experiment was performed with triplicate cell cultures for each condition. Lanes 1-3 starting from the left of both blots were loaded with lysates from control cultures that were not virally transduced. Lanes 4-6 were loaded with lysates from cells that were transduced with the DeltaOcc adenovirus. Lanes 7-9 at the right of both blots were loaded with lysates from cells that were transduced with the GFP only control adenovirus. The proteolytic, active forms are labeled at the left of each blot

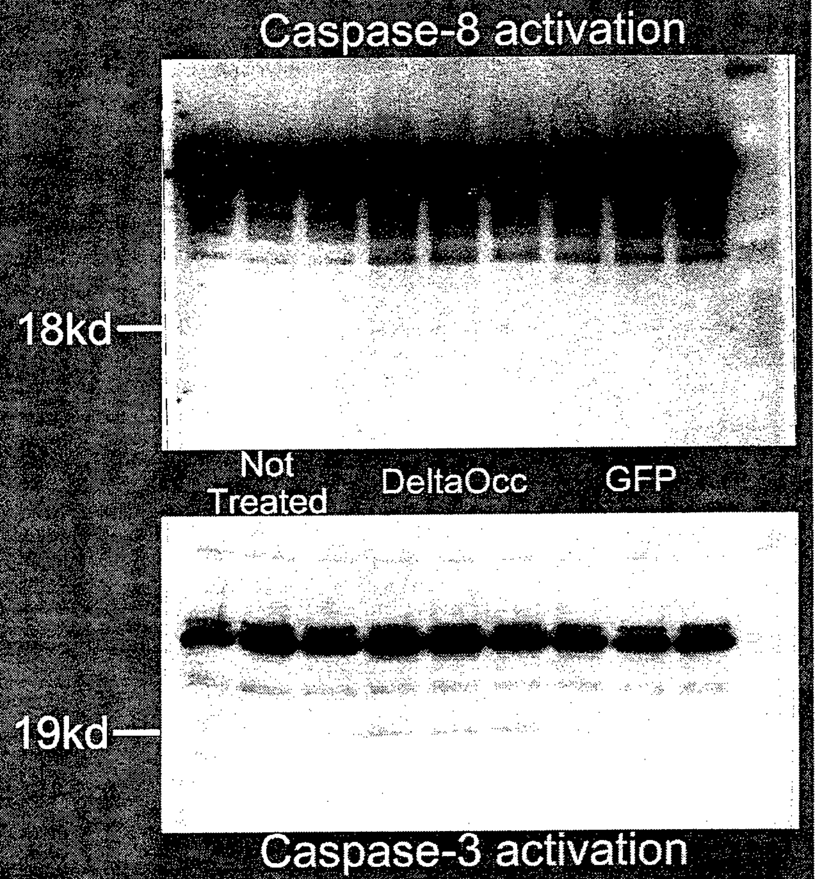
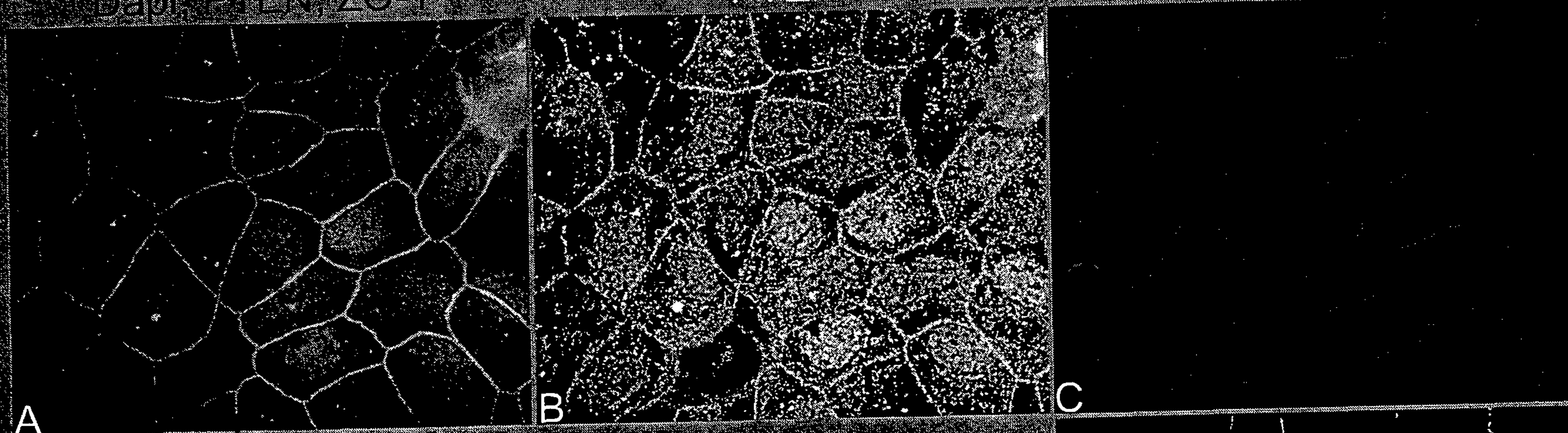
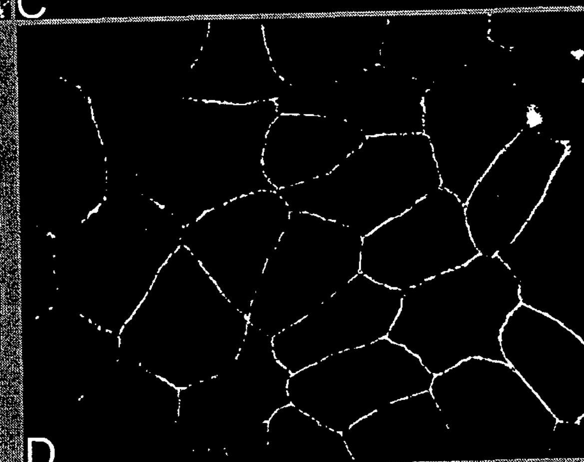


Figure 7: On page 6 in body of report.

Figure 8: PTEN localized with ZO-1 at the tight junction of mammary epithelial cells.





Confluent CIT3 cells were fixed and stained with antibodies against PTEN and ZO-1. The same cells are shown in each of the four panels. Panel A shows dapi nuclear staining in blue, PTEN antibody staining in green, and ZO-1 staining in red. Panel B shows PTEN antibody staining in white. Panel C shows ZO-1 antibody staining in red. Panel D shows a digitally rendered mask that only displays pixels beyond threshold intensity for <u>both</u> ZO-1 and PTEN.